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Six-transmembrane epithelial antigen of prostate 3 promotes hepatic insulin resistance and steatosis

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome characterized by excessive deposition of fatty acids in the liver. Further deterioration leads to nonalcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma, creating a heavy burden on human health and the social economy. Currently, there are no effective and specific drugs for the treatment of NAFLD. Therefore, it is important to further investigate the pathogenesis of NAFLD and explore effective therapeutic targets for the prevention and treatment of the disease. Six-transmembrane epithelial antigen of prostate 3 (STEAP3), a STEAP family protein, is a metalloreductase. Studies have shown that it can participate in the regulation of liver ischemia-reperfusion injury, hepatocellular carcinoma, myocardial hypertrophy, and other diseases. In this study, we found that the expression of STEAP3 is up-regulated in NAFLD. Deletion of STEAP3 inhibits the development of NAFLD in vivo and in vitro, whereas its overexpression promotes palmitic acid/oleic acid stimulation-induced lipid deposition in hepatocytes. Mechanistically, it interacts with transforming growth factor beta-activated kinase 1 (TAK1) to regulate the progression of NAFLD by promoting TAK1 phosphorylation and activating the TAK1- c-Jun N-terminal kinase (JNK)/p38 signaling pathway. Taken together, our results provide further insight into the involvement of STEAP3 in liver pathology.

Keywords: STEAP3, metalloreductase, NAFLD, Glucose metabolism disorder, lipid deposition, Hepatic steatosis, Hepatocytes, PA/OA, TAK1, JNK/p38 signaling pathway

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prominent cause of chronic liver disease worldwide (1). The many associated hepatic and extrahepatic comorbidities result in a serious public health burden of NAFLD, which is estimated to affect approximately 25% of the world’s adult population (2). Continuous lipid accumulation and inflammation are considered basic characteristics of NAFLD progression from simple steatosis to nonalcoholic steatohepatitis (3), which may eventually lead to liver cirrhosis and hepatocellular carcinoma (HCC) (4). Due to sharply increasing morbidity, the discovery of treatments and drugs for these diseases is urgently required (5). In recent years, various molecular targets, which are involved in NAFLD pathogenesis, have been identified (6). However, effective pharmacological therapies for NAFLD are still lacking (7,8).

The six-transmembrane epithelial antigen of prostate (STEAP) family proteins, consisting of four multi-transmembrane proteins, have been reported to affect metal homeostasis via the reduction and uptake of iron and copper (9). Among these proteins, STEAP3 is the major endosomal ferrireductase that participates in various biological
processes including molecular trafficking, cell proliferation, apoptosis, and inflammation (10-12). STEAP3 null mice show microcytic anemia caused by abnormal erythroid maturation, which is thought to be the result of ablation of the ferrireductase of STEAP3 (13). Exosome secretion is also severely compromised in STEAP3 deficient mice via the p53-dependent secretory pathway (14). p53-induced STEAP3 promotes apoptosis and negatively regulates G2/M transition (12). Moreover, STEAP3 deficiency is responsible for the impaired Toll-like receptor 4 (TLR4)-mediated inflammatory response in macrophages (11). Additionally, STEAP3 acts as a regulator of inflammation and apoptosis in liver ischemia-reperfusion injury (15). However, the functions of STEAP3 in NAFLD remain poorly understood.

In the current study, we identified a significant increase of STEAP3 expression in the liver of NAFLD mice and hepatocytes of a cell model. The deficiency of STEAP3 relieves glucose metabolic disorders and hepatic steatosis in high-fat diet (HFD)-induced mice and palmitic acid/oleic acid (PA/OA)-stimulated primary hepatocytes. In contrast, hepatocytes overexpressing STEAP3 exhibited enhanced lipid accumulation and upregulated gene expression for lipid metabolism. Systematic investigation revealed that inhibition of the mitogen-activated protein kinase (MAPK) cascade is strongly associated with the deletion of STEAP3 in fatty liver. Furthermore, STEAP3 interacts with transforming growth factor beta–activated kinase 1 (TAK1), a pivotal member of the MAPK signaling pathway in hepatocytes. Additionally, inhibition of TAK1 rescued the NAFLD phenotypes induced by STEAP3 overexpression. Collectively, our results indicate that the absence of STEAP3 inhibits the progression of NAFLD by physically interacting with TAK1 and activating the MAPK signaling cascade.

Methods & Materials

Animals
Eight-weeks male mice of 20-26 g were included in this study. All mice used for the experiment were housed in a specific pathogen-free animal laboratory with a controlled environment that included temperature (24±2°C), humidity (40-70%), and a 12 h light-dark cycle. The NAFLD model was established by feeding mice a HFD for 24 weeks (HFD containing 20% protein, 60% fat, and 20% carbohydrate; H10060, Huafukang, Beijing, China). Mice in the control group were fed normal chow diet (1010086, Xietong Biology, Jiangsu, China). Water was available ad libitum for all mice in all the experiments. All animal protocols were approved by the Animal Care and Use Committee of the Renmin Hospital of Wuhan University. STEAP3-KO mice were purchased from the Texas A&M Institute for Genomic Medicine (IST13594C11; TIGM, Germany).

Physiological parameters detection
The weight and blood glucose levels of the mice were measured after fasting for 6 h every four weeks. Blood insulin levels were estimated using an enzyme-linked immunosorbent assay kit (Cloud-Corp, Wuhan, China). Homeostatic model assessment of insulin resistance (HOMA-IR) was evaluated using the following formula: \([\text{fasting blood glucose (mmol/L) } \times \text{fasting serum insulin (mIU/L)}] / 22.5\). Glucose tolerance test (GTT) assays were performed at the 23rd week as described previously: animals were injected (i.p.) at a dose of 1 g/kg after 6 h of fasting. Blood glucose levels were measured at baseline and at 15, 30, 60, and 120 min after glucose injection, and the area under the curve was calculated using the conventional trapezoid rule. Levels of serum lipid triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured using an ADVIA 2400 Chemistry System Analyzer (Siemens, Tarrytown, NY, USA).

**Histological analysis**
Liver sections cut from formaldehyde-fixed and paraffin-embedded liver tissues of experimental mice in a thickness of 5 μm were used for hematoxylin-eosin (H&E) staining (Hematoxylin, G1004, Servicebio Co., Ltd., Wuhan, China; Eosin, BA-4024, Baso Co., Ltd., Zuhuai, China) to evaluate the morphological change of liver tissue and lipid accumulation, and periodic acid-Schiff (PAS) staining (Periodic acid, BA-4044B, Schiff reagent, BA-4044A, Baso, Zuhuai, China) to evaluate the accumulation of glycogen. Frozen liver sections cut from liver tissues embedded in optimum cutting temperature compound with a thickness of 8 μm were used for Oil Red O staining (O0625, Sigma, St. Louis, MO, USA) for fat content observation. After staining, histological images were captured using a light microscope (ECLIPSE 80i, Nikon, Tokyo, Japan) for all staining sections.

**Isolation of primary hepatocytes and in vitro TAK1 inhibitor experiment**
Murine primary hepatocytes were isolated from 6-8-week old male mice. After anaesthetization, the mice were perfused with liver perfusion medium at 37°C until the liver turned khaki. The liver was perfused with liver digestion medium containing collagenase IV. After digestion, the livers were removed and the liver capsules were opened to release the hepatocytes into the medium and then filtered through a 70 μm steel mesh. Hepatocytes were obtained by centrifugation at 50 g twice. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained in an incubator with 5% CO₂. PA and OA stimulation (PO) was used to construct a hepatocyte NAFLD model in vitro. For oil red O staining, the cells were treated with 0.2 μM PA and 0.4 μM OA for 12 h, and for western blot and RT-PCR detection, the cells were treated with 0.5 μM PA and 1.0 μM OA for 12 h. For the in vitro TAK1 inhibitor experiment, primary hepatocytes were treated with 2 μM TAK1 inhibitor, 5Z-7-oxozaenol (O9890-1 MG, Sigma, St. Louis, MO, USA), for 12 h.
Adenovirus infection
Adenoviruses carrying sequences encoding mouse Steap3 (AdSteap3) and hairpin-forming oligonucleotides targeting Steap3 (AdshSteap3) were purchased from Hanbio Biotechnology (Shanghai, China). Recombinant adenoviruses were plaque-purified using caesium chloride density gradient centrifugation and verified by restriction digestion. Similar adenoviral vectors that did not carry the Steap3 gene (Adcontrol) and scrambled short hairpin RNA (AdshRNA) were used as controls. After purification, the recombinant adenoviruses were diluted to a titre of $10^{10}$ plaque-forming units (PFU) per milliliter.

Cellular Nile red staining
Primary hepatocytes on climbing pieces were treated with PA and OA and then fixed with 4% paraformaldehyde for Nile red staining. Primary hepatocytes were stained with 1 µM Nile red (22190; Fanbo Biochemicals Co., Ltd., Beijing, China) at room temperature for 20 min. The nuclei were stained with 4',6-diamidino2-phenylindole (DAPI). Images were obtained using a confocal laser scanning microscope system (TCS SP8, Leica, Wetzlar, Germany).

RNA extraction and quantitative RT-PCR analysis
Total RNA was extracted from liver tissues or hepatocytes using TRIzol reagent in the phenol-chloroform method and dissolved in diethyl pyrocarbonate (DEPC)-treated water. Subsequently, 1-2 µg of RNA was reverse-transcribed to obtain complementary DNA (cDNA). Quantitative real-time PCR assays were performed with cDNA and specific primers using a real-time PCR system (LightCycler 480 Instrument II) with β-actin as the reference gene. Primer sequences are listed in Supplementary Table 1.

Western blot
Tissues and cells were lysed with radio immunoprecipitation assay lysis buffer (65 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with protease inhibitor. Proteins were separated by high-speed centrifugation and quantified using a bicinchoninic acid protein assay kit. Equivalent amount of protein samples were transferred to polyvinylidene fluoride (PVDF) after separating them on 6-10% SDS-PAGE. The membranes were blocked for 1 h at room temperature and sequentially incubated with specific primary antibodies overnight at 4°C, and the corresponding secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The membranes were washed with TBST three times after blocking and incubation. Finally, chemiluminescence signals were detected and visualized using the ChemiDoc MP Imaging System (Bio-Rad). The antibodies used are listed in Supplementary Table 2.

Co-immunoprecipitation assay
L02 cells transfected with the indicated plasmids were cultivated for 24 h and lysed in
cold immunoprecipitation lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; and 1% Nonidet P-40). After high-speed centrifugation at 4°C, the supernatant containing the protein was incubated with protein G agarose beads and IgG or anti-tag antibodies at 4°C overnight. Beads were collected by centrifugation at 3000 rpm at 4°C and washed three times with 150 mM or 300 mM NaCl buffer. Proteins were eluted from the beads with SDS loading buffer and boiled at 95°C for 5–10 min prior to SDS-PAGE and Western blot analysis.

**RNA sequencing and Transcriptome analysis**
Total RNA extracted from the liver tissue was used for RNA sequencing with MGISEQ 2000. Differentially expressed genes were identified using two standards: a fold change greater than 2 and the corresponding adjusted P values less than 0.05. The RNA sequencing data has been uploaded to the public database of NCBI (PRJNA900784).

**Hierarchical clustering**
Hierarchical clustering creates a hierarchical nested clustering tree by calculating the similarity between different samples, using the weighted average distance (unweighted pair-group method with arithmetic means, UPGMA) algorithm, and the HCLUST function of R language for visualization.

**Gene set enrichment analysis**
Gene set enrichment analysis (GSEA) uses the gene sets in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway to sort genes according to the level of differential expression and subsequently tests whether the gene sets are concentrated at the top or bottom of the sequencing list to obtain the overall expression changes of these gene sets. This analysis was performed on the Java GSEA (Version 3.0) platform using the "Signal2Noise" metric, and gene sets with P values less than 0.05 and false discovery rate (FDR) less than 0.25 were considered statistically significant.

**KEGG pathway enrichment analysis**
KEGG is a comprehensive database that integrates genomic, chemical, and systematic functional information. KEGG pathway enrichment analysis was performed for all differentially expressed genes using Fisher's exact test, and KEGG pathway annotations of all genes in the reference genome were downloaded from the KEGG database. Pathways with P values less than 0.05 were defined as significantly enriched pathways.

**Statistical analysis**
Data are shown as the mean and standard deviation (mean ± SD). SPSS 21 was used for the statistical analysis. First, the normality of the data was analyzed. For data conforming to the normal distribution, the two-tailed Student’s t-test was used for comparative analysis between the two groups, and a one-way analysis of variance (ANOVA) was used for comparative analysis of three or more groups. Specifically, the
Bonferroni test was used for groups with variance homogeneity and Tamhane's T2 analysis was used for groups with variance heterogeneity. Non-parametric tests were used for data that did not conform to normal distribution. Statistical significance was set at $P < 0.05$.

**Results:**

**STEAP3 is upregulated in NAFLD mice and cell models**
To investigate the association between STEAP3 and NAFLD, we determined STEAP3 expression in HFD-induced NAFLD mouse model. Compared with normal controls, STEAP3 levels upregulated significantly in liver tissues of the NAFLD group (Figure 1A). The same phenomenon was observed in genetically obese (ob/ob) mice (Figure 1B). Consistent with the results in mouse models, STEAP3 levels increased in primary hepatocytes after PA/OA treatment compared with that in cells treated with BSA (Figure 1C). Taken together, these findings suggest that STEAP3 levels are increased in lipid-laden hepatocytes.

**STEAP3 promotes lipid accumulation in hepatocytes**
Given that the expression level of STEAP3 is up-regulated in NAFLD, we overexpressed STEAP3 in primary hepatocytes using adenovirus infection to study its effect on NAFLD in vitro. We first confirmed the overexpression of STEAP3 (Figure 2A). PA/OA stimulation was used to establish cell models, followed by Nile red staining; we found that STEAP3 overexpression promoted PA/OA-induced lipid accumulation in the hepatocytes (Figure 2B). Consistently, RT-PCR revealed that the mRNA levels of fatty acid synthesis and transport-related genes Acaca, Fads2, Fasn, and Cd36 were upregulated in STEAP3-overexpressed hepatocytes following PA/OA treatment (Figure 2C). In contrast, when we knockdown STEAP3 in primary hepatocytes by adenovirus infection (Figure 2D), opposite results were obtained, as evidenced by the reduced lipid accumulation and decreased mRNA levels of lipid metabolism-related genes (Figure 2E, 2F). Taken together, our findings suggest that STEAP3 promotes lipid accumulation in the hepatocytes.

**Steap3 deficiency inhibits glucose metabolic disorder induced by HFD**
We used Steap3 systemic gene knockout (KO) mice to investigate the function of Steap3 deletion in NAFLD; wild-type (WT) mice were used as controls. An HFD for 24 weeks was used to establish an NAFLD model. We first confirmed the Steap3 deficiency using Western blot analysis (Figure 3A). Normal chow (NC) fed Steap3-KO and WT mice exhibited comparable metabolic characteristics (Figure 3B-3G). However, after 24 weeks of HFD, the fasting body weights, fasting blood glucose levels, serum insulin content, and HOMA-IR of the KO mice were lower than those of the WT mice (Figure 3B-3E). In addition, the GTT assay proved that the loss of Steap3 significantly enhanced the glucose tolerance induced by the HFD (Figure 3F, 3G). To
further confirm the abnormality of glucose metabolism, we performed PAS staining of liver sections and found enhanced glycogen accumulation in KO-HFD mice (Figure 3H). In line with the results mentioned above, the RT-PCR assay revealed downregulation of G6pc and Pck1 mRNA levels, indicating that Steap3 deficiency inhibits gluconeogenesis (Figure 3I, 3J). Collectively, Steap3 deletion leads to resistance of diet-induced obesity.

**Steap3 knockout relieves HFD-induced hepatic steatosis**
Besides glucose metabolic disorder, KO mice showed lower liver weight, liver weight/body weight ratio, serum TG, and total TC contents than that of WT mice after HFD, whereas these parameters did not differ clearly during NC feeding (Figure 4A-4D). Similarly, H&E and Oil Red O staining demonstrated reduced hepatic lipid accumulation and steatosis in KO-HFD mice (Figure 4E, 4F). Further RT-PCR results revealed that the mRNA levels of fatty acid synthesis and transport-related genes, including Srebp-1c, Acaca, Fads2, Fasn and Cd36 decreased significantly, whereas β-oxidation-related gene Cpt1a was upregulated (Figure 4G-4L), confirming the effect of Steap3 deletion in alleviating lipid metabolic disorder. Additionally, KO-HFD mice showed lower serum levels of ALT and AST than that of WT-HFD mice (Figure 4M, 4N). Collectively, these results revealed that Steap3 knockout mitigated the development of NAFLD.

**STEAP3 significantly regulates glucose metabolism and lipid metabolism related pathways**
To profile the differences in gene expression resulting from Steap3 deletion during NAFLD, the livers of Steap3-KO and WT mice after 24 weeks of HFD were subjected to RNA-Seq analysis. Through principal component analysis, the samples from KO and WT mice were separated into two clusters based on their gene expression similarities (Figure 5A). GSEA showed that glucose metabolism and lipid metabolism response-related pathways were inactive due to Steap3 deficiency (Figure 5B). Heat maps of transcriptome analysis showed decreased activation of lipid metabolism-related (Figure 5C) and glycometabolism-related (Figure 5D) genes in the Steap3-KO group.

**STEAP3 interacts with TAK1 and activates the TAK1-JNK/P38 signaling pathway**
To further elucidate the underlying mechanism of the effect of STEAP3 on NAFLD, KEGG pathway enrichment analysis was performed and results showed that the MAPK pathway was the most significantly enriched signaling pathway (Figure 6A). This indicated that the MAPK signaling pathway might be involved in the function of STEAP3 in NAFLD. To further elucidate the underlying effect of STEAP3 on MAPK pathway activation, we examined the activation of MAPK-related molecules in HFD-induced animal and PA/OA-stimulated cell models. Western blotting results showed that phosphorylation of JNK and p38 in the liver tissues of HFD Steap3-KO mice was inhibited, while total JNK, p38, ERK1/2, and phosphorylated ERK1/2 remained
unchanged (Figure 6B). Similar changes were observed in primary STEAP3 knock down hepatocytes stimulated by PA/OA (Figure 6C). However, STEAP3 overexpression in PA/OA-treated primary hepatocytes exacerbated the phosphorylation of JNK and p38 but not ERK1/2 (Figure 6D). As upstream regulatory molecules of MAPK signaling pathway, TAK1 and ASK1 have been reported to be involved in the activation regulation of JNK and p38(16). Therefore, the levels of total and phosphorylated TAK1 and ASK1 in the above samples were determined, and the results showed that STEAP3 promoted the phosphorylation of TAK1 (Figure 6E-6G). In addition, STEAP3 has been reported to interact with TAK1 during hepatic I/R injury(17). We overexpressed STEAP3 in primary hepatocytes and showed that STEAP3 interacts with TAK1 under PA/OA stimulation (Figure 6H). Altogether, our results demonstrate that STEAP3 interacts with TAK1 and regulates the TAK1-JNK/p38 signaling pathway in NAFLD.

**TAK1 inhibition abolishes the regulation of STEAP3 overexpression on lipid accumulation in hepatocytes**

To further explore whether STEAP3 regulation of NAFLD depends on TAK1 activation, we used an inhibitor of TAK1 phosphorylation (iTAK1) in PA/OA-stimulated STEAP3 overexpression primary hepatocytes. TAK1 inhibitor significantly inhibited the phosphorylation of TAK1, JNK and p38. And the up-regulated phosphorylation of TAK1, JNK and p38 induced by STEAP3 overexpression were eliminated by TAK1 inhibitor (Figure 7A). Nile red staining showed that, compared with the DMSO-treated control, the accumulation of lipids induced by STEAP3 overexpression was abolished by iTAK1 (Figure 7B). Moreover, upon iTAK1 treatment, the mRNA level of lipid metabolism-related genes upregulated by STEAP3 overexpression was attenuated significantly, indicating that iTAK1 could reverse lipid metabolism disorder induced by STEAP3 overexpression (Figure 7C). Collectively, our results suggest that STEAP3 knockout alleviates the development of NAFLD via the TAK1-JNK/p38 signaling pathway.

**Discussion**

NAFLD has become the fastest growing liver disease worldwide over the past two decades and is predicted to be the leading cause of end-stage liver disease and liver transplantation (18,19). NAFLD progression can be followed by the development of fibrosis, liver cirrhosis, liver failure, and HCC (20-22). As a result of lipid metabolism disorders, the presence and accumulation of lipid droplets in over 5% of hepatocytes is known to be the predominant hallmark of NAFLD (23). In the present study, we provide evidence that the absence of STEAP3 inhibits diet-induced NAFLD by improving glucose metabolism disorders and lipid accumulation in hepatocytes. With respect to mechanisms, STEAP3 knockout relieves metabolic disorder via interaction with TAK1.
to suppressive MAPK signaling pathway.

NAFLD is a liver disease closely related to abnormal glucose and lipid metabolism, and most patients with NAFLD have glucose metabolism disorders (24). Long-term overnutrition can cause insulin resistance in the liver, resulting in glucose metabolism damage (25,26). In addition, insulin resistance can inhibit the hydrolysis of triglycerides in vivo, increase the decomposition of peripheral adipose tissue, and allow a large number of free fatty acids to enter the liver through the portal vein system, resulting in fatty liver (27-29). Studies have shown that HFD-induced insulin resistance and gluconeogenesis can regulate the progression of NAFLD (30-32). STEAP family proteins are unique to mammals and share innate activities as metalloredoxases. In previous studies, STEAP family proteins were found to be involved in metabolic diseases, such as insulin resistance and obesity (33-35). For example, STEAP4 promotes insulin-stimulated glucose uptake and regulates insulin sensitivity in mature human adipocytes (34). However, the function of the STEAP family proteins in NAFLD remains unknown. In this study, STEAP3 was found to be involved in the regulation of HFD-induced glucose metabolism and liver lipid deposition, which proved the regulatory role of STEAP family proteins in NAFLD for the first time.

The MAPK signaling pathway has been reported to play a prominent role in the regulation of metabolism in the liver (36). Fatty liver activates stress-responsive MAPKs such as p38 MAPKs and JNKs (37,38). TAK1, a key upstream regulator of MAPK cascade activation, is a MAPK kinase family serine threonine kinase that plays a critical role in the regulation of autophagy, inflammation, and lipid metabolism (39). TAK1 can hyperactivate downstream JNK and NF-κB signaling, which has been proven to promote the development of steatohepatitis (32). Conversely, degradation of activated p-TAK1 ameliorates NAFLD in a mouse model (40). However, TAK1 deficiency also leads to severe hepatosteatosis and HCC (41). Because of the dual effect of TAK1 on NAFLD, inhibition of hyperactivation of TAK1 without abolishing its physiological function may be a potential therapeutic treatment for NAFLD (39). Here, we demonstrated for the first time that STEAP3 interacts with TAK1 to regulate the phosphorylation level of TAK1 and thus the progress of NAFLD, suggesting that STEAP3 has the potential to be a novel therapeutic target for NAFLD.

This study has some limitations. Systematic, instead of cell type-specific, STEAP3 knockout mice were used to construct an in vivo NAFLD model. However, the use of global KO mice in this study increases the difficulty of determining the effect of STEAP3 on body weight gain after fed HFD for 24W which needs to be verified in the future with liver-specific knockout mice. But, Liver-specific knockout or over expression had also been reported to affect body weight in mice induced by HFD treatment(42,43). The hepatocyte-specific effects of STEAP3 knockout in pathological NAFLD must be thoroughly elucidated in hepatocyte-specific knockout mice.
In conclusion, our study revealed that the interaction between STAEP3 and TAK1 enhances the activation of the MAPK signaling pathway. STEAP3 deficiency protected against diet-induced NAFLD including glucose and lipid accumulation in the liver tissue by inhibiting phosphorylation of TAK1, JNK and P38. Importantly, these findings may provide new insights into the mechanism of NAFLD and a new approach for the development of an effective treatment of NAFLD by targeting STEAP3.

**Data Availability**

The data sets that support the findings of this study are available in this paper or the Supplementary Information.
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Figure legend

Figure 1. STEAP3 is upregulated in NAFLD mice and cell models. (A) Representative western blot analysis and quantitative results of STEAP3 protein expression in liver tissue of NC control or HFD mice. n = 4 mice in each group. (B) Representative western blot analysis and quantitative results of STEAP3 protein expression in liver tissue of lean or obese (ob/ob) mice. n = 4 mice in each group. (C) Representative western blot analysis and quantitative results of STEAP3 protein expression in primary hepatocytes treated with BSA or PA/OA. n = 4 independent experiments. Statistical analysis was carried out by two-tailed Student’s t-test. **, P < 0.01.

Figure 2. STEAP3 promotes lipid accumulation in hepatocytes. (A) Representative western blot results for STEAP3 overexpression detection in the indicated groups. (B) Representative images of Nile red staining of control and STEAP3 overexpression primary hepatocytes treated with BSA or PA/OA. (C) Representative RT-PCR analysis results of lipid metabolic genes Acaca, Fads2, Fasn, and Cd36 in the indicated groups. (D) Representative western blot results for STEAP3 knockdown detection in the indicated groups. (E) Representative images of Nile red staining in the indicated groups. (F) Representative RT-PCR analysis results of lipid metabolic genes Acaca, Fads2, Fasn, and Cd36 in the indicated groups. For A, B, D, and E, n=3 independent experiments. For C and F, n=4 independent experiments. Statistical analysis was carried out by two-tailed Student’s t-test. **, P < 0.01.

Figure 3. STEAP3 deficiency inhibits glucose metabolic disorder induced by HFD (A) Representative western blot results of STEAP3 in liver tissue of WT and STEAP3-KO mice. n=3 mice in each group. (B-E) Representative fasting body weights (B), fasting blood glucose levels (C), fasting insulin levels (D), and HOMA-IR indices (E) of KO mice and their littermate controls at the indicated time points in response to HFD feeding. n=8-10 mice in each group. (F, G) Representative GTT assays and AUC detection results of WT and KO mice fed with NC or HFD for 23 weeks. n=8-10 mice in each group. (H) Representative PAS staining images of liver sections in the indicated groups. bar=100 μm, n=6-7 mice in each group. (I, J) Representative RT-PCR analysis of glucose metabolic gene G6pc and Pck1 mRNA levels in liver of HFD WT and KO mice. n=4 in each group. For B to G, statistical analysis was carried out by one-way ANOVA. For I and J, statistical analysis was carried out by two-tailed Student’s t-test. *, P < 0.05 and **, P < 0.01 vs. WT NC group. #, P < 0.05 and ##, P < 0.01 vs. WT HFD group.

Figure 4. STEAP3 knockout relieves HFD-induced hepatic steatosis. (A, B) Liver weight (A) and liver weight/body weight ratio (B) of WT and KO mice fed with NC or
HFD for 24 weeks. n=8-10 mice in each group. (C, D) Serum TG and TC levels in WT and KO mice fed with NC or HFD for 24 weeks. n=8-10 mice in each group. (E, F) Representative H&E (E) and Oil red O (F) staining images of liver section from HFD WT and KO mice. bar=100 μm, n = 6-7 mice in each group. (G-L) Representative RT-PCR analysis of lipid metabolic genes Srebp-1c, Acaca, Fads2, Fasn, Cd36, and Cpt1a mRNA levels in liver of HFD WT and KO mice. n=4 in each group. (M, N) Serum ALT and AST levels in WT and KO mice fed with NC or HFD for 24 weeks. n=8-10 mice in each group. For A to D, M, and N, statistical analysis was carried out by one-way ANOVA. For G to L, statistical analysis was carried out by two-tailed Student’s t-test. *, P < 0.05 and **, P < 0.01 vs. WT NC group. #, P < 0.05 and ##, P < 0.01 vs. WT HFD group.

Figure 5. STEAP3 significantly regulates glucose metabolism and lipid metabolism related pathways. (A) unsupervised hierarchical clustering analysis of the RNA-seq data from the mice fed with HFD for 24 weeks. (B) GSEA pathway enrichment analysis of pathways related to lipid metabolism and glycometabolism. (C, D) Heat map of lipid metabolism–related and glycometabolism-related gene expression profiles based on the RNA-seq data set. n=5 mice per group.

Figure 6. STEAP3 interacts with TAK1 and regulates the TAK1-JNK/P38 signaling pathway. (A) KEGG pathway enrichment analysis based on the RNA-seq data set. (B-D) Representative Western blot analysis of protein levels of total and phosphorylated ERK, JNK, and p38 in liver of WT and KO mice fed with HFD for 24 weeks (B), PA/OA-stimulated STEAP3-knockout primary hepatocytes (C) and PA/OA-stimulated STEAP3-overexpressed primary hepatocytes (D). (E-G) Representative Western blot analysis of protein levels of total and phosphorylated TAK1 in the indicated groups. (H) Representative Co-IP analysis of TAK1 and STEAP3 in PA/OA-stimulated hepatocytes. n=3 independent experiments.

Figure 7. TAK1 inhibition abolishes the regulation of STEAP3 overexpression on lipid accumulation in hepatocytes. (A) Representative Western blot analysis of STEAP3, total and phosphorylated TAK1, JNK, and p38 in control and STEAP3 overexpression primary hepatocytes treated with DMSO or iTAK1. n=3 in each group. (B) Representative images of Nile Red staining of control and STEAP3 overexpression primary hepatocytes treated with DMSO or iTAK1. (C) Representative RT-PCR analysis of lipid metabolic genes Acaca, Fads2, Cd36, and Fasn in control and STEAP3 overexpression primary hepatocytes treated with DMSO or iTAK1. n = 4 independent experiments in each group. Statistical analysis was carried out by one-way ANOVA. *, P < 0.05 and **, P < 0.01 vs. Control DMSO group. #, P < 0.05 and ##, P < 0.01 vs. STEAP3 DMSO group.
Figure 1

A. NC-24W vs HFD-24W

B. Lean vs ob/ob

C. BSA vs PO
Figure 2

A

B

C

D

E

F

Figure 2

A

B

C

D

E

F
Figure 4

(A) Liver weight (g) compared between WT and KO mice on NC and HFD diets. 

(B) LW/BW (%) comparison between WT and KO mice on NC and HFD diets. 

(C) Serum TG (mmol/L) comparison between WT and KO mice on NC and HFD diets. 

(D) Serum TC (mmol/L) comparison between WT and KO mice on NC and HFD diets. 

(E) H&E staining of liver sections from WT-HFD and KO-HFD mice. 

(F) Oil red O staining of liver sections from WT-HFD and KO-HFD mice. 

(G) Srebp-1c mRNA levels in WT and KO mice on HFD. 

(H) Acaca mRNA levels in WT and KO mice on HFD. 

(I) Fads2 mRNA levels in WT and KO mice on HFD. 

(J) Fasn mRNA levels in WT and KO mice on HFD. 

(K) Cd36 mRNA levels in WT and KO mice on HFD. 

(L) Cpt1a mRNA levels in WT and KO mice on HFD. 

(M) Serum ALT (U/L) comparing NC and HFD groups. 

(N) Serum AST (U/L) comparing NC and HFD groups.
Figure 5

A

B

C

D

WT HFD KO HFD

Glycerolipid metabolism
Fatty acid biosynthesis
Sphingolipid metabolism
Phospholipase D signaling pathway
Fatty acid elongation
Sphingolipid signaling pathway
Biosynthesis of unsaturated fatty acids
Other glycan degradation
Insulin secretion

Classify
Lipid metabolism
Glycometabolism

GSEA NES

Elov15
Elov17
Pppla3
Acot2
Acox5
Acox5
Scd5
Scd2
Gpat3
Scd3
Scd1
Scd2
Gpat3
Scd3
Scd1
Elov15

WT HFD KO HFD

Relb
Rac2
Cdc25b
Clec4n
Rras
Lpl
Cd14
Agpat4
Pak1
Tslp
Flna
Cdc42
Arnb2
Dusp2
Rps6ka4
Rras2
Grk3
Acsf4
Rps6ka5
Thbs1
Nos1
Naip3
Moqat1
Pla2g4a
Pak2
Runx3
Psip1
Runx1
Cilia
Naip6
Oas1a
Csf3r
Cftr
Gbpm
Ncf4
Acox3
Crab5
Csf2rb2

glycocometabolism related genes

2 1 0 -1 -2

2 1 0 -1 -2

2 1 0 -1 -2
Figure 6

A

B

C

D

E

F

G

H

Journal Pre-proof
**Author Contributions**

Y.Z. supervised and supported the whole work. W.C. supervised the design, experiments, data analysis and manuscript preparation. T.D. S.C and W.X. carried out the main work. Z.L. and J.T. participated in the experiments. Y.Y. and B.D. participated in data analysis.